SHORT COMMUNICATION

Electron Microscopic Studies of Lipopolysaccharides from Phase I and Phase II *Coxiella burnetii*

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Lipopolysaccharides from phase I (LPSI) *Coxiella burnetii* Ohio and Nine Mile strains and from phase II (LPSII) Nine Mile strain were stained negatively and positively and examined with the electron microscope. The ultrastructure of LPSI and LPSII positively stained with uranyl formate or uranyl acetate was ribbon-like. When negatively stained with uranyl acetate, LPSI was ribbon-like but LPSII exhibited hexagonal lattice structures. However, LPSII stained negatively with sodium phosphotungstate and ammonium molybdate exhibited hexagonal lattice ultrastructures which were not identical to those observed when negatively stained with uranyl acetate. The hexagonal lattice structures formed *in vitro* were due to the interactions of LPSII and the staining reagents rather than to protein–LPS interactions. The differences in the ultrastructures of LPSI and LPSII are undoubtedly based on variations in their chemical composition.

INTRODUCTION

Coxiella burnetii, the rickettsial agent of Q fever, undergoes a transition from virulent (phase I) to avirulent (phase II) organisms with a concomitant change in smooth and rough lipopolysaccharide (LPS) chemotype (Schramek & Mayer, 1982; Amano & Williams, 1984). LPS extracted into the aqueous phase with hot (68 °C) phenol from phase II cells (LPSII) is composed of neutral sugars (mannose and glyceromannoheptose), phosphate, glucosamine, ethanolamine and 3-deoxy-D-manno-octulaseonic acid (KDO)-like materials. LPS from phase I cells (LPSI) contains, in addition to the LPSII components, glucose, many unidentified components, and a unique disaccharide, galactosaminuronyl α -(1→6)-glucosamine (Amano & Williams, 1984).

Previous studies of the ultrastructure of LPS obtained from wild-type and rough mutants of *Salmonella minnesota* showed marked differences depending upon the extraction procedure and contaminating chemical components (Amano & Fukushima, 1984*a, b, c*). In the present study, we compare the ultrastructures of negatively and positively stained LPSs from phase I and phase II *C. burnetii* using electron microscopy, since differences in chemical composition of LPSs determine their ultrastructures. We show that the hydrophobic LPSII has the ability to form a hexagonal lattice structure in the absence of contaminating outer membrane components.

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Abbreviations: CBOI, *C. burnetii* strain Ohio phase I; CB9MI, *C. burnetii* strain Nine Mile phase I; CB9MII, *C. burnetii* strain Nine Mile phase II; UA, uranyl acetate; PTA, sodium phosphotungstate; AM, ammonium molybdate; UF, uranyl formate.

METHODS

Organisms. *C. burnetii* strains Ohio phase I (CBOI), Nine Mile phase I (CB9MI), and Nine Mile phase II (CB9MII) were cultivated and purified as described previously (Williams *et al.*, 1981).

LPS extraction and purification. LPSs from the various strains were extracted by hot (68 °C) phenol/phosphate-buffered saline (pH 7.2) (Williams *et al.*, 1981), and dialysed extensively (7 d) against daily changes of demineralized distilled water. LPSs were further purified by ultracentrifugation and the pellets were washed twice more as described by Amano & Williams (1984). The LPSs were not exposed to divalent cations during the purification and electrodialysis was not done.

Staining and microscopy. LPSs suspended (1 mg ml⁻¹) in demineralized distilled water were negatively stained by mixing with an equal volume of staining reagents [3% (w/v) uranyl acetate (UA), 3% (w/v) sodium phosphotungstate (PTA), or 2% (w/v) ammonium molybdate (AM)] and deposited directly on carbon-coated collodion grids for electron microscopic observation (Amano & Fukushima, 1984*a, b, c*). Positive staining was done by applying LPSs directly to the grids. After drying the LPSs were stained with 1% (w/v) uranyl formate (UF) and 1% (w/v) UA (Amano & Fukushima, 1984*a, b, c*). All specimens were examined with a Hitachi electron microscope, type HU-12As, operating at an accelerating voltage of 100 kV.

RESULTS AND DISCUSSION

The images of LPSI and LPSII positively stained with UF showed ribbon-like structures (Fig. 1*a, b*). This observation is in agreement with the reports of Chan *et al.* (1976) and Baca *et al.* (1980) who described ultrastructures of positively stained LPSI and LPSII as ribbon-like. These structures are similar to positively stained LPS from smooth strains of *Salmonella* (Amano & Fukushima, 1984*a, b, c*).

Negative staining of LPSs with UA revealed marked differences between LPSI and LPSII. LPSI was ribbon-like (Fig. 1*c*), while LPSII appeared as sheets having an ordered hexagonal lattice structure (Fig. 1*d*), resembling the ultrastructure observed in mixtures of the outer membrane protein O-8 and LPS from *Escherichia coli* K12 (Yamada & Mizushima, 1980). The LPS lattice structure obtained by Yamada & Mizushima (1980) required protein O-8 in combination with LPS; neither component alone formed the lattice structure. In addition, the LPSII lattice structures were very similar to the structure of acetic acid-treated LPS from a *Yersinia enterocolitica* rough mutant as described by Acker & Wartenberg (1976). Since the protein content of the LPSII fractions from *C. burnetii* was only 0.6% (Amano & Williams, 1984), it is unlikely that the lattice formation of LPSII was due to interaction with protein.

LPSII negatively stained with PTA (Fig. 1*g*) and AM (Fig. 1*h*) exhibited hexagonal ultrastructures that were different from those of LPSII stained negatively with UA (Fig. 1*d*). These ultrastructures suggest that LPS-reagent interactions produce ordered hexagonal lattice structures with different aggregations. The ultrastructure of LPSII negatively stained with AM also showed hole-like structures (Fig. 1*h*) which resemble the porin present in the outer membrane of Gram-negative bacteria (Nikaido, 1979). On the other hand, the ultrastructure of LPSI from CB9MI negatively stained with PTA showed ribbon-like structures having two different widths (approximately 8 and 3 nm) (Fig. 1*e, f*). The 3 nm ribbon-like structure may be one filament whereas the 8 nm wide structure may be a double-track filament. The differences in ultrastructure between LPSI and LPSII are undoubtedly based on the differences in chemical composition between the two LPSs (Amano & Williams, 1984). However, the marked differences in ultrastructure between positively stained and negatively stained LPSII cannot be accounted for by this known chemical composition. Also, the chemical interactions which produce the periodic arrays observed in negatively stained LPSII are intriguing, since they are not produced by contaminating outer membrane proteins.

The LPSs from Gram-negative bacteria are composed predominantly of two moieties, a complex heteropolysaccharide moiety and a lipid A moiety. These LPSs form bilayer membranes in aqueous solutions (Shands, 1971; Shands *et al.*, 1967) with the polysaccharide moiety facing outwards. The hydrophilic and hydrophobic properties of phase I and phase II LPS, respectively (Schramek & Mayer, 1982; Amano & Williams, 1984; Schramek *et al.*, 1985) suggest that the carbohydrate side chain contributes to the physico-chemical differences. Both LPSs from *C. burnetii* also formed the bilayer structures as shown in Fig. 1(*a, b, c*). A comparison

Fig. 1. Electron micrographs of LPSs purified from phase I and phase II *C. burnetii* stained positively with UF (*a*, *b*), and negatively with UA (*c*, *d*), PTA (*e*, *f*, *g*) and AM (*h*). (*a*), (*e*) and (*f*), LPSI from CB9MI; (*c*), LPSI from CBOI; (*b*), (*d*), (*g*) and (*h*), LPSII from CB9MII. Bars, 0.1 μ m.

of the ultrastructures of *Salmonella* wild-type and R mutant LPSs with those of phase I and phase II LPSs indicates marked differences (Amano & Fukushi, 1984*a, b, c*). The molecular interactions which induce the ultrastructures observed in this study are unknown. However, *C. burnetii* LPSs contain hydrophobic moieties composed of fatty acids and other unidentified compounds which apparently behave much like hydrophobic protein-LPS interactions. The fatty acid contents of LPSI and LPSII are very low (0.6–2.2 and 1.2%, respectively) (Amano & Williams, 1984) compared to the fatty acid content of the LPS of Gram-negative bacteria (over 10%). Recent studies on the chemical components constituting the hydrophobic moieties of LPSII suggest some unidentified compounds which differ from common fatty acids (K.-I. Amano & J. C. Williams, unpublished data).

Divalent cations such as Mg^{2+} interact with acidic LPS to facilitate the formation of the hexagonal lattice structures of R-form LPS of *Klebsiella* species (Kato *et al.*, 1985). Although we did not subject our LPS preparations to electrodialysis to remove cations and basic materials we extracted LPSs using cells suspended in PBS. Since we have not done an elemental analysis of our LPS preparation we cannot speculate on the contribution of cations to the production of the hexagonal lattice structure produced by negative staining. It would be interesting to study the ultrastructure of *C. burnetii* LPSs in various salt forms. Further studies are necessary to determine the complete chemical composition of *C. burnetii* LPSI and LPSII and the relationship between the ultrastructures of their various salt forms (Kato *et al.*, 1985), their immunological properties, and their contributions to the functional and structural rigidity of the outer membrane of this obligate intraphagosomal parasite.

REFERENCES

- ACKER, G. & WARTENBERG, K. (1976). Ultrastructure of lipopolysaccharides of *Yersinia enterocolitica*, *Salmonella typhimurium* and *Escherichia coli*. *Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene (Abteilung I, Originale A)* **235**, 439–452.
- AMANO, K. & FUKUSHI, K. (1984*a*). Chemical and ultrastructural differences in endotoxic glycolipids from *Salmonella typhimurium* R mutant extracted with various solvent systems. *Microbiology and Immunology* **28**, 135–148.
- AMANO, K. & FUKUSHI, K. (1984*b*). Chemical and ultrastructural comparison of endotoxins extracted from *Salmonella minnesota* wild type and R mutants. *Microbiology and Immunology* **28**, 149–159.
- AMANO, K. & FUKUSHI, K. (1984*c*). Electron microscopic studies of endotoxins treated with alkaline and acid reagents. *Microbiology and Immunology* **28**, 161–168.
- AMANO, K. & WILLIAMS, J. C. (1984). Chemical and immunological characterization of lipopolysaccharides from phase I and phase II *Coxiella burnetii*. *Journal of Bacteriology* **160**, 994–1002.
- BACA, O. G., MARTINEZ, I. L., ARAGON, A. S. & KLASSEN, D. (1980). Isolation and partial characterization of a lipopolysaccharide from phase II *Coxiella burnetii*. *Canadian Journal of Microbiology* **26**, 819–826.
- CHAN, M. L., MCCHESENEY, J. & PARETSKY, D. (1976). Further characterization of a lipopolysaccharide from *Coxiella burnetii*. *Infection and Immunity* **13**, 1721–1727.
- KATO, N., OHTA, M., KIDO, N., ITO, H., NAITO, S. & KUNO, T. (1985). Formation of a hexagonal lattice structure by an R-form lipopolysaccharide of *Klebsiella* sp. *Journal of Bacteriology* **162**, 1142–1150.
- NIKAIDO, H. (1979). Nonspecific transport through the outer membrane. In *Bacterial Outer Membranes: Biogenesis and Functions*, pp. 361–407. Edited by M. Inouye. New York: John Wiley.
- SCHRAHEK, S. & MAYER, H. (1982). Different sugar compositions of lipopolysaccharides isolated from phase I and phase II cells of *Coxiella burnetii*. *Infection and Immunity* **38**, 53–57.
- SCHRAHEK, S., RADZIEJEWSKA-LEBRECHT, J. & MAYER, H. (1985). 3-C-Branched aldoses in lipopolysaccharide of phase I *Coxiella burnetii* and their role as immunodominant factors. *European Journal of Biochemistry* **148**, 455–461.
- SHANDS, J. W. JR (1971). Evidence for bilayer structure in gram-negative lipopolysaccharide: relationship to toxicity. *Infection and Immunity* **4**, 167–172.
- SHANDS, J. W. JR, GRAHAM, J. A. & NATH, K. (1967). The morphologic structure of isolated bacterial lipopolysaccharide. *Journal of Molecular Biology* **25**, 15–21.
- WILLIAMS, J. C., PEACOCK, M. G. & MCCAUL, T. F. (1981). Immunological and biological characterization of *Coxiella burnetii*, phases I and II, separated from host components. *Infection and Immunity* **32**, 840–851.
- YAMADA, H. & MIZUSHIMA, S. (1980). Interaction between major outer membrane protein (O-8) and lipopolysaccharide in *Escherichia coli* K12. *European Journal of Biochemistry* **103**, 209–218.